SPECIFICATION

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Peptide for Regulation of Urokinase Plasminogen Activator and Method of Optimizing Therapeutic Efficacy

Field of Invention

[0001] This invention discloses a peptide comprising of six amino acids (EEIIMD) having the property to bind at the "docking"site in urokinase plasminogen (uPA) activator and tissue type plasminogen activator (tPA) outside the active site. The invention also relates to the regulation of activity when uPA or tPA is given in treatment of ischemic stroke, in particular to the single chain urokinase plasminogen activator (scuPA) to clear blood clots that cause stroke or myocardial infarction or induce intracerebral hemorrhage (ICH).

Background to the Invention

[0002] Pro-urokinase (Pro-UK) also known as single chain urokinase plasminogen activator (scuPA), is a naturally occurring molecule released from vascular endothelial cells in response to formation of blood clots and other pathological conditions. ScuPA or Pro-UK can be activated by two different mechanisms a) by cleavage of a single peptide bond by plasmin that leads to the generation of the active form composed of two chains (tcuPA) and b) by binding of scuPA to its receptor, urokinase plasminogen activator receptor (uPAR).

[0003] Plasminogen activator inhibitor type 1 (PAI-1) binds to tcuPA and inhibits its catalytic activity. However, PAI-1, which binds tcuPA with high affinity, binds with only low affinity, if at all, to scuPA.

[0004] Plasminogen activator inhibitor type 1 interacts with both tissue PA and uPA and inhibits the catalytic activity of both proteins. PAI-1, which binds tPA and uPA with high affinity is present at high concentrations in the circulation of patients suffering from hypertension. And, reduction of blood pressure by medical treatment results in a decrease of PAI-1 concentrations. The underlying mechanism of action for the increase of PAI-1 in certain pathological conditions is not understood well. However, the inverse relationship with tPA and/or uPA suggests that PAI-1 serves to neutralize in some way the vasoactive effect of tPA and/or uPA. *Simmons M, Cardiol. Clin* 1995, 13:339-345; *Cipolla M et al., Stroke,* 2000, 31:940-945; of PAI-1; and *Higazi, A.A.-R et al., J. Biol. Chem.,* 1995, 270:9472-9477.

[0005] Tissue-type plasminogen activator is the only therapy for acute thromboembolic stroke, which is approved by the Food and Drug Administration (FDA). However, there is reason for concern that use of tPA for treatment of ischemic stroke may expose patients to secondary intracerebral hemorrhage. *Wardlaw JC et al, Lancet* 1997, 350:607-614. This is because there is an approximately six percent incidence of subsequent symptomatic intracerebral hemorrhage and approximately fifty percent of these patients die. The appearance of intracerebral hemorrhage after treatment with tPA is attributed to its capacity to interfere with the normal vasoactivity of the cerebral blood vessels.

[0006] Several approaches to thrombolytic therapy have been under investigation, one being through the systemic infusion of activators of the naturally occurring or commercially produced recombinant varieties of fibrinolytic agents. Urokinase is a thrombolytic agent active through the conversion of plasminogen to plasmin. Urokinase is a complex protein of unknown structure which is found in urine in trace amounts. Recombinant forms of urokinase have been developed and are being tested for clinical efficacy, for example U.S. Patent 4, 558,010 issued to Abbott Laboratories, describes a recombinant deoxyribonucleic acid which codes for the plasminogen activator protein having human urokinase activity.

[0007]

In a co-pending United States Application Serial No. 09/902,135, it was found that a six amino acid peptide EEIIMD, could reduce the undesirable side effects of fibrinolytic agents, for example, the risk of intracerebral hemorrhage in patients

receiving tPA, uPA, tcuPA, streptokinase, rt-PA or alteplase, rt-PA derivatives or anisoylated streptokinase complex. In the protocol employed, the peptide was introduced into the thrombolytic regimen in later stages to prevent the vasoactive or side effects of the primary thrombolytic agent.

[0008] The question of whether the peptide has any effect when administered in the early stage of the thrombolytic therapy, has not been investigated heretofore. The present invention describes some unexpected results obtained when the peptide is administered when combined with a plasminogen activa tor right from the start of the thrombolytic therapy. The results are unexpected because they demonstrate a synergistic effect when the peptide and the plasminogen activator are administered together in in vitro and in in vivo systems. The present invention thus provides novel compositions of different plasminogen activators and the peptide and methods for optimizing the efficacy of thrombolytic agents in combination therapeutic regimens. Such an approach suggests that the effective dosage of the thrombolytic agent can be reduced in the presence of the peptide. This in turn reduces the risk for side effects of these agents, the side effects being manifested in the late stage of therapy.

Summary of Invention

[0009] The present invention relates to the compositions and use of a polypeptide composed of 6 amino acids EEIIMD, in combination with one or more thrombolytic agents including, but not limited to, scuPA, tPA, uPA, tcuPA, streptokinase, rt-PA or alteplase, rt-PA derivatives (such as reteplase, lanoteplase and TNK-rt-PA), anisoylated plasminogen streptokinase complex (APSC) or anistreplase, or streptokinase derivative.

[0010] More specifically, the polypeptide is useful in enhancing the activity of the thrombolytic agent (including, but not limited to, scuPA, tPA, uPA, tcuPA, streptokinase, rt-PA or alteplase, rt-PA derivatives (such as reteplase, lanoteplase and TNK-rt-PA), anisoylated plasminogen streptokinase complex (APSC) or anistreplase, or streptokinase derivative, and thereby reducing the effective dosage of the thrombolytic agent required in the prevention and/or treatment of thromboembolic disorders.

[0011] Also, contemplated by the present invention are methods of reducing the occurrence of intracerebral hemorrhage in patients receiving fibrinolytic therapy , including, but not limited to, scuPA, tPA, uPA, tcuPA, streptokinase, rt-PA or alteplase, rt-PA derivatives (such as reteplase, lanoteplase and TNK-rt-PA), anisoylated plasminogen streptokinase complex (APSC) or anistreplase, or streptokinase derivative.

[0012] In yet another embodiment, the present invention is directed to pharmaceutical kits for the treatment of thromboembolic disorders in mammals, the kits comprising a sterile container of a thrombolytic agent (including, but not limited to, scuPA, tPA, uPA, tcuPA, streptokinase, rt-PA or alteplase, rt-PA derivatives (such as reteplase, lanoteplase and TNK-rt-PA), anisoylated plasminogen streptokinase complex (APSC) or anistreplase, or streptokinase derivative, and the peptide in commercially available forms, both in amounts therapeutically effective to treat the thromboembolic disorders.

[0013] The foregoing kits may include, thrombolytic agents if desired, (including, but not limited to, scuPA, tPA, uPA, tcuPA, streptokinase, rt-PA or alteplase, rt-PA derivatives (such as reteplase, lanoteplase and TNK-rt-PA), anisoylated plasminogen streptokinase complex (APSC) or anistreplase, or streptokinase derivative, in amounts therapeutically effective to treat thromboembolic disorders as well as prevent any side effects.

[0014] It is also within the scope of this invention to provide kits, where appropriate, of combinations of two or more thrombolytic agents along with the peptide. It is further the object of the present invention to provide methods of treating thromboembolic disorders using a conjunctive therapy in combination with one or more of fibrinolytic agents including scuPA, tPA, uPA, tcuPA, streptokinase, rt-PA or alteplase, rt-PA derivatives (such as reteplase, lanoteplase and TNK-rt-PA), anisoylated plasminogen streptokinase complex (APSC) or anistreplase, or streptokinase derivative, the method comprising of administering the combination therapy right from the start of the regimen.

Brief Description of Drawings

[0015] The advantages and features of the present invention will become readily apparent after reading the following detailed description and referencing the drawings, which are:Fig. 1A is a diagram describing the results of experiments on the effect of tPA on PE-induced contraction of isolated rat aorta rings. Contraction of aortic rings was induced by increasing the concentrations of phenylephrine (PE), in the absence of tPA (full triangles) or in the presence of 1nM (filled squares) or 20nM tPA (empty squares).

[0016] Fig. 1B describes the results of experiments in which the contraction of aortic rings was induced in the absence of TNK-tPA (filled triangles), in the presence of 1nM tPA(filled squares) or 20nM tPA(empty squares).

[0017] Fig. 2 is a graphical representation of the results obtained in experiments to study the effect of PAI-1 on the vasoactivity of tPA. The EC50 of PE was determined in the absence (Control) or presence of 1nM tPA, 20nM tPA, 1nM tPA and an equimolar concentration of PAI-1, 20nM tPA and an equimolar concentration of PAI-1, 1nM tPA and 2 μ M or EEIIMD or 20nM tPA and 2 μ M of EEIMD.

[0018] Fig. 3 is a graphical representation of the results obtained in experiments to study the effect of RAP and anti-LRP antibodies on the vasoactivity of tPA. The EC50 of PE was determined in the absence (Control) or presence of 1nM tPA, 20nM tPA, 1nM tPA and an equimolar concentration of PAI-1, 20nM tPA and an equimolar concentration of PAI-1, 1nM tPA and 2 μ M or EEIIMD or 20nM tPA and 2 μ M of EEIMD.

Detailed Description

[0019] In accordance with the present invention, pharmaceutical compositions of the peptide and one or more plasminogen activators are provided, such compositions having an enhancing effect on inducing lysis of blood clots but an inhibitory effect on causing side effects related to hemorrhagic disorders that result with some fibrinolytic agents. Also, contemplated by the present invention are methods of improving the efficacy of fibrinolytic agents and reducing the occurrence of intra-cerebral hemorrhage in patients receiving fibrinolytic agents in the treatment of thromboembolic disorders.

[0020] The present invention also provides pharmaceutical compositions and kits comprising of the polypeptide EEIIMD in combination with one or more of fibrinolytic

agents including tPA, uPA, tcuPA, streptokinase, rt-PA or alteplase, rt-PA derivatives (such as reteplase, lanoteplase and TNK-rt-PA), anisoylated plasminogen streptokinase complex (APSC) or anistreplase, or streptokinase derivatives.

[0021] The present invention also provides methods for improving the efficacy of fibrinolytic agents, thereby reducing the effective dosage, by combining the fibrinolytic agent with the peptide, in a ratio of 0.1/1.0 to 1.0/0.1 respectively.

[0022] The present invention further provides methods for preventing and/or treating side effects such as intracerebral hemorrhage and related vascular abnormalities associated with fibrinolytic agents such as scuPA by providing therapeutic regimens of scuPA alone or in combination, in combination with an effective amount of the peptide to prevent and/or inhibit side effects.

[0023] TPA is a single-chain serine protease composed of 530 amino acids, although originally 527 were identified. The t-PA enzyme is composed of several domains with homologies to other proteins: a) a finger domain comprising residues 4-50, b) a growth factor domain comprising residues 50-87, and c) two kringles comprising residues 87-176 and 176-262, and the protease domain constituted by residues 276-527 comprising the catalytic triad. Initial binding of t-PA to fibrin is governed by the finger domain and by kringle 2, which binds to exposed carboxyl-terminal lysine residues. TPA has a weak affinity for plasminogen in the absence of fibrin (Km = 76uM) but a much higher affinity in the presence of fibrin (K between 0.15 and 1.5 μ M). In this reaction plasminogen binds to fibrin primarily via specific structures called the "lysine-binding site." Thus one way of regulating fibrinolysis is at the level of plasminogen activation localized at the fibrin surface.

[0024] Plasminogen activator inhibitors, specifically PAI-1 and PAI-2 inhibit the physiological plasminogen activators, for example, PAI-1 is the primary inhibitor of t-PA and u-PA in plasma. PAI-1, a serine protease inhibitor, is a single chain glycoprotein derived from endothelial cells and other cell types. PAI-1 inhibits tPA by the formation of a complex between the active site of tPA and the "bait"residues (Arg 346-Met 347) of PAI-1.

[0025]

The PAI-1 concentration in plasma is increased in several diseases, including

venous thromboembolism, obesity, sepsis and coronary artery disease. High PAI-1 activity constitutes an independent risk factor for myocardial infarction in young subjects within three (3) years of the first attack. There is a clear correlation between the circadian variation in the time of onset of myocardial infarction, with the highest incidence at about 8 am. The circadian rhythm of plasma PAI-1 activity is also highest early in the morning.

[0026] Plasminogen activator inhibitor type 1 interacts with both tPA and tcuPA and inhibits the catalytic activity of both proteins. PAI-1, which binds to tPA with high affinity (Heckman CM, Archires of Biochem Biophysics, 1988, 262:199-210), is also present at high concentrations in the circulation of patients suffering from hypertension. Reduction of blood pressure by medical treatment results in the decrease of PAI-1 concentration. *Erden YC et al. AmJ Hypertens,* 1999, 12:1071-1076. The underlying mechanism of action to explain the increase of PAI-1 in some pathological conditions is not understood. PAI-1 also binds to tcuPA and inhibits its catalytic activity, but PAI-1 has a low affinity for binding scuPA.

[0027] For example, PAI-1 reacts with single chain tPA, two chain tPA and tcuPA. The second-order rate constant for their inhibition of single-chain tPA by PAI-1 is about 10^{7} M^{-1} ,while inhibition of two chain tPA and tcuPA is somewhat faster. Positively charged regions in tPA (residues 296-304) and uPA (residues 179-184) are involved in this rapid reaction. PAI activity is very rapidly cleared from the circulation by the liver. Except for platelets, which contain both functional and inactive PAI-1, PAI-1 is not stored within cells, but is rapidly and constitutively secreted after synthesis.

[0028] PAI-1 binds tPA and uPA through two independent epitopes, one of which interacts with the active site. The other epitope is composed of 6 amino acid residues, EEIMP, that correspond to the amino acid residues 350 to 355 of PAI-1. This second epitope of PAI-1 interacts with a binding "docking"site in uPA and tPA that is outside of the active site. *Adams DS et al., J. Biol. Chem,* 1999, 266:8476-8482.

[0029] The present invention describes the effect of the 6 amino acid peptide on the fibrinolytic activity of scuPA and indicates that the peptide stimulates synergistically the activity of scuPA on blood clot lysis. These observations are described in detail in the Examples section.

[0030] The peptide of the present invention, while preventing and/or inhibiting the adverse effects of scuPA on blood vessels, has no effect on the fibrinolytic activity of scuPA. The peptide is therefore useful in clot lysis during thrombolytic therapy in myocardial infarction, stroke and related complications.

[0031] The commercially available tPA is produced by recombinant DNA technology (such as recombinant t-PA, rt-PA) in two forms: a single-chain preparation (alteplase) and a double-chain preparation (dute plase). Other tPA types include reteplase (r-PA) and a mutant of rt-PA, TNK-rt-PA. See below for details under section entitled "TNA=t-PA and rtPA".

[0032] The preferred dosage regimen of fibrin-selective alteplase consists of a weight-adjusted accelerated (front-loaded) regimen over 90 minutes (15 mg bolus, 0.75 mg/kg over 30 minutes (not to exceed 50 mg) and .05 mg/kg over 60 minutes [not to exceed 35 mg]). The present invention provides a composition of alteplase and the peptide, such that the level of fibrinolytic activity achieved in above dosage regimen is actually obtained with much lower dosage of alteplase. This is because the combination of alteplase and the peptide results in better lysis activity.

[0033] The above improvement is also observed when the preferred dosage regimen of fibrin-selective alteplase consists of a weight-adjusted accelerated (front-loaded) regimen over 90 minutes (15 mg bolus, 0/75 mg/kg over 30 minutes (not to exceed 50mg] and .05 mg/kg over 60 minutes [not to exceed 35mg])

[0034] The preferred dosage regimen for the peptide consists of an amount effective to optimally enhance the activity of the fibrinolytic activity while also preventing the harmful vasoactive effects of a fibrinolytic agent on a case by case basis. The peptide may be a component of a sequence of varying numbers of amino acids, or the peptide may have a modification of one or more amino acids in its sequence. The ratio of peptide/tPA, UPA, or TNK-tPA may be in the range of 0.1/1.0 to 1.0/0.1.

[0035]

The peptide of the present invention is useful in treatment of sepsis, when administered alone in an effective dosage or in combination with traditional anti-coagulant therapy. Under physiological conditions, several antithrombotic mechanisms act in concert to prevent clotting, and to preserve blood fluidity. Any

thrombin that escapes the surveillance of this physiological anticoagulant system is available to convert fibrinogen to fibrin. This in turn triggers the fibrinolytic system.

[0036] TNK-tPA and rtPA

[0037] T-PA consists of five domains: a fibronectin finger-like domain, an epidermal growth factor domain (EGF), two kringle domains (K1 and K2), and a protease domain. TNK-t-PA differs from rtPA in the K1 and protease domains. In K1 the glycosylation site at amino acid 117 (N117) has been shifted to amino acid 103, while in the protease domain there is a tetra-alanine substitution (K296A/H297A/R298A/R299A) in the plasminogen activator inhibitor-1 (PAI-1) docking site that makes it resistant to inactivation by PAI-1.

[0038] TNK-tissue plasminogen activator (TNK-tPA) is a bioengineered variant of tissue-type plasminogen activator (t-PA), having a longer half-life than tPA. It is resistant to inactivation by plasminogen activator inhibitor-1 on account of having a tetra-alanine substitution in the protease domain (K296A/H297A/R298A/R299A).

[0039] TNK-tPA exhibits 80-fold higher resistance to plasminogen activator inhibitor-1 (PAI-1) than tPA and 14 fold greater relative fibrin specificity.

[0040] In vitro, TNK-tPA is 8 and 13 fold more potent than tPA towards whole blood and platelet-enriched clots, respectively.

[0041] In vivo, the time required by TNK-tPA for 50% lysis in arterial venous shunt models of fibrinolysis in rabbits, was only one third of that required by rtPA. In spite of these enormous advantages of TNK-tPA over tPA in experimental situations, TNK-tPA has no significant advantage over tPA in clinical studies.

[0042] In comparative clinical trials, TNK-tPA is found to have equivalent efficacy to rtPA and with rate of intracranial hemorrhage similar to that with rtPA. The unique significant advantage of TNK-tPA over rtPA is the fact that TNK-tPA is associated with fewer non-cerebral bleeding episodes (4.66% vs. 5.94%).

[0043] The present invention elucidates the basis of the discrepancy between the in vitro effects of TNK-tPA and the in vivo effects in humans. Specifically, the effects of TNK-tPA, rtPA and/or tPA were examined on the PE-induced contraction of isolated rings.

Results obtained are described in detail below in the section on EXAMPLES. Briefly, results obtained indicate that rtPA has two binding epitopes that are involved in vasoactivity. The first epitope has greater affinity (around 1nM) and inhibits the PE induced vasoconstriction. The second epitope has a lower affinity (around 20nM) and stimulates the PE-induced vasoconstriction. The present invention also suggests that the first epitope that induces prodilatation, has been inactivated in TNK-tPA.

[0044] Results obtained in the present invention indicate that the vasoactive effect of TNK-tPA is unaffected by equimolar concentration of PAI-1 peptide. However, at 5 molar concentration of the peptide, the vasoactive effect of TNK-tPA was abolished. Thus, results described in the EXAMPLES suggest that the PAI-1 derived hexapeptide EEIIMD is useful for inhibiting the vasoactive effects of tPA, rtPA and/or TNK-tPA.

[0045] TPA and LRP

[0046] TPA is known to bind (Strickland JHaDK.LRP:a multifunctional scavenger and signaling receptor. *J. Clin. Invest.* 2001;108:779-784)(LRP). This binding is regulated by PAI-1. Results obtained in the present invention demonstrate that LRP is also involved in the vasoactivity of tPA (see below for details in section on EXAMPLES).

[0047] Specifically, anti-LRP antibodies and the LRP antagonist, recombinant receptor associated protein, rRAP, both abolished the vasoactive effect of tPA and TNK-tPA. Results described in the present invention suggest that the anti-LRP antibodies and/or rRAP prolong the half-life of tPA in the circulation. These results also suggest that anti-LRP antibodies and/or RAP may be used to prolong the half-life of scuPA or scuPA/suPAR complex (described in a co-pending U.S. applications Serial Nos. 09/325,917, filed June 4, 1999; 09/968,752, filed October 2, 2001; 09/302,392, July 10, 2001; and 09/902,135, filed July 10, 2001; and incorporated herein, by reference, in its entirety.)

[0048] EXAMPLE 1

[0049] The effect of TNK-tPA on PE-induced contraction was compared with the effect of tPA, in the isolated aorta rings. The experimental procedure followed has been described earlier (Haj-Yehia A, Nassar T, Sachais B, Kuo A, Bdeir.K., Al-Mehdi A-B, Mazar A, Cines D, Higazi AA-R. Urokinase-derived peptides regulate vascular smooth

muscle contraction i vitro and in vivio. *FASEB J.* 2000;14:1411-1422.

[0050] Fig. 1A shows that 1nM tPA inhibited PE-induced vasoconstriction. Fig. 1B shows that at the same concentration (1nM) TNK-tPA exerted an opposite effect to that of tPA on the contraction of aorta rings. 1nM of TNK-tPA stimulated the vasoconstriction induced by PE.

[0051] Since the concentration of tPA used in the previous experiments was in the physiological range, but was much below the therapeutic range, the effect of higher concentrations of tPA variants on vasoactivity was examined. Fig. 1A shows that increasing the concentration of rtPA produced a similar effect to that induced by 1nM TNK-tPA. 20nM of rtPA stimulated the constriction induced by PE and the EC50 was decreased from 34 to 1.6nM.

[0052] Fig. 1B shows that increasing the concentration of TNK-tPA from 1 to 20nM increased its stimulatory effect on PE-induced vasoconstriction, by decreasing its EC50 from 34 to 0.63 nM.

[0053] EXAMPLE 2

[0054] In an attempt to understand the basis for the modification in the vasoactivity of TNK-tPA, the role of the PAI-1 docking site in the process was examined. Fig. 2 shows that the rtPA pro-vasodilatation as well as pro-vasoconstrictive effects are inhibited by equimolar concentrations of PAI-1.

[0055] PAI-1 interacts with tPA through independent sites; the catalytic site and a docking site, present in the amino acids 296 to 299. The PAI-1 docking site is mutated in TNK-tPA. To examine in greater detail the role of the PAI-1 docking site in the vasoactivity of TNK-tPA specifically and of rtPA in general, we examined the effect of the PAI-1 derived hexapeptide EEIMD that correspond to the amino acid residues 350 to 355 of PAI-1 (the epitope in PAI-1 that interacts with the tPA docking site (Madision EL, Goldsmith EJ, Gerard RD, Gething MJH, Sambrook JF, Bassel-Duby RS. Amino acid residues that affect interaction of tissue plasminogen activator with plasminogen activator inhibitor 1. *Proceedings of the National Academy of Science, USA.* 1990;87:3530-3534. Madison EL, Goldsmith EJ. Gething M-J, H., Sambrook JF, Gerard RD. Restoration of serine protease-inhibitor interaction by protein engineering.

Journal of Biological Chemistry. 1990;265:21423-21426.

[0056] Fig. 2 shows that a concentration of 2 μ M, the PAI-1 derived peptide abolished the vasoactive effects of rtPA. Interestingly, the vasoactive effect of TNK-tPA was unaffected by 2 μ M concentration of PAI-1 peptide. However, at 10 μ M, the peptide abolished the effect of TNK-tPA.

[0057] The present invention therefore provides a means of inhibiting the vasoactivity of both tPA and TNK-tPA by combining them with an effective amount of the PAI-1 peptide.

[0058] EXAMPLE 3

[0059] The effect of revertase and TNK-tPA on the PE induced vasocontraction was studied in presence or absence of the LRP antagonist (RAP) or anti LRP antibodies. The results obtained shown in Figure 3, indicate that the vasoactive effect of tPA and/or TNK-tPA is totally abolished by the anti-LRP antibodies as well as by the LRP antagonist rRAP.

[0060] The present invention is not to be limited in scope by the embodiment disclosed in the example which is intended as an illustration of one aspect of the invention and any methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

[0061] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, any equivalents to the specific embodiments of the invention described herein. All references and co-pending applications cited herein are incorporated by reference in their entirety in the present specification. Such equivalents are intended to be encompassed by the claims.